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RESEARCH ARTICLE

DETECTION OF MICRONUCLEI IN GILL CELLS AND HAEMOCYTES OF FRESH WATER SNAILS EXPOSED TO MERCURIC CHLORIDE

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ABSTRACT

The present study was undertaken to evaluate the toxic potential of the heavy metal adequately present in agro-industrial effluents viz. mercury using *in vivo* micronucleus assay in a fresh water snail, *Bellamya bengalensis* (2n=22). Gill cells and haemocytes of the mollusc species were subjected to four sub-lethal concentrations of heavy metal compound $HgCl_2$ (4.0µg/l, 8.0µg/l, 16.0µg/l, 32.0µg/l) for 24, 48, 72 and 96h of exposure periods. Significant increase in the frequency of micronuclei was observed in molluscs exposed to the metal compound as compared to the control. These findings depicted the genotoxic potential of mercuric chloride by using sub-lethal concentrations on fresh water snails.

INTRODUCTION

In environmental genotoxicity indication system, the micronucleus test has served as an index of cytogenetic damage for over 30 years. Micronucleus consists of acentric fragments of chromosomes or whole chromosomes which are not incorporated into daughter nuclei at anaphase. These small nuclei can be formed as a consequence of the lagging of a whole chromosome (aneugenic event) or acentric chromosome fragments (clastogenic event) (Heddle, 1973; Schmid, 1975). A Micronucleus arises in cell divisions due to spindle apparatus malfunction, the lack or damage of centromere or chromosomal aberrations (Fenech, 2000). These micronuclei are appropriate indices of pollutant exposure because it is a quick, sensitive and reliable analysis to determine damage to the DNA (Dixon *et al.,* 2002). Micronuclei assay as cytogenetic damage has been carried out for genotoxicity of Benzopyrene (Venier *et al.,* 1997; Siu *et al.,* 2004), PAH (Leonard and Hellou, 2001; Francoini *et al.,* 2005), Heavy metals (Al-Sabti, 1994; Bolognesi *et al.,* 1999) and oil pollution in field (Barsiene, 2002; Barsiene *et al.,* 2006) or laboratory (Reid and McFarlen, 2003) study.

Many contaminants exert their effects via genotoxic and metabolically toxic mechanisms, simultaneously causing carcinogenesis, embryotoxicity and inflict long term damage to organisms (Jha *et al.,* 2000). Since the aquatic environment is the ultimate recipient of the pollutants produced by natural and anthropogenic sources, accumulation and persistence of heavy metals in the aquatic environment constitute a threat to biological life (George, 1990; Fleeger *et al.,* 2003). Aquatic heavy metal pollution usually represents high levels of Hg, Cr, Pb, Cd, Cu, Zn, Ni etc. in water system. Mercury is considered as one of the most dangerous of the heavy metals because of its high toxicity, bioaccumulative properties, and other deleterious effects on biota including genetic alteration or mutagenesis (WHO, 1990). The biggest sources of mercury pollution are chloride-alkaline industry, mining and use of mercury derivatives and the atmospheric residues coming from garbage burning and fossil fuels also contribute to this pollution (Weiner *et al.,* 2003). It is a non-biodegradable heavy metal which undergoes biomagnification during its transfer through different trophic levels in food chain.

A category of special concern heavy metals have induced several types of ill effects in target and non-target organisms and other animals. In the aquatic invertebrate, Beaby and Eaves (1983), observed that molluscs can accumulate higher concentration of metal ions than other groups of invertebrates. These are most sensitive of all the aquatic animals towards the pollutants passing through the river from adjoining settlement industries. The accumulation of toxic material become hazardous to the aquatic organisms and to surrounding human population because the molluscs are most important factor of

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food web in both terrestrial and aquatic communities. These biomarkers provide an integrated detection system for environmental pressures and can be used as an early warning signal of environmental deterioration (Bolognesi *et al*., 1999; Osman *et al*., 2007). In the present study, *in vivo* genotoxic effects of mercuric chloride on gill cells and haemocytes of fresh water snail, *Bellamya bengalensis* (2n=22) were investigated by using the micronucleus test.

MATERIALS AND METHODS

Animals

Fresh water snail, *B. bengalensis* was used as an experimental animal. Healthy adult live specimens measuring 23-26 mm in shell height and 2.8-3.5 gms in weight were collected and brought to the laboratory in wide mouthed plastic containers containing natural water without any stress and mechanical injuries. The snails were subjected to repeated washings with tap water and were then transferred to plastic troughs containing aerated water for acclimatization to the laboratory conditions for 7 days. They were fed with diatoms and filamentous green algae. Feeding was stopped 24h prior to toxicity tests and molluscs were not fed during experimental period.

Test chemicals used

Mercuric chloride dissolved in distilled water at a concentration of four sub-lethal doses as 4.0µg/l, 8.0µg/l, 16.0µg/l and 32.0µg/l was used to conduct genotoxicity test against the snails. 96h LC_{50} value determined by the method suggested by Finney (1980) was found to be 36.0 µg/l for mercuric chloride.

Experimental design

After acclimatization, snails were grouped into five sets. One set maintained in normal dechlorinated tap water served as the control group and the remaining four sets of animals were used for treatment. The experimental groups of animals were continuously exposed to four different concentrations of mercuric chloride viz. 4.0µg/l, 8.0µg/l, 16.0µg/l and 32.0µg/l for 24, 48, 72 and 96h of duration. The molluscs of control group were also exposed for the same duration of time. Only, haemolymph and gill epithelium were used for performing the micronucleus test.

Slides preparation

Haemolymph (haemocytes) and gill cells are the target tissues most frequently considered for micronuclei determination in molluscs (Venier *et al*., 1997). Haemolymph was collected with a hypodermic syringe which was inserted in foot through the operculum. The pressure was applied by withdrawing the plunger of the syringe and needle was slowly moved deeper until drops of pale blue haemolymph were aspirated. Thin smears of haemolymph were prepared on clean glass slides, air dried and fixed in methanol for 10 minutes. Slides were then stained in working solution of 4% Giemsa stain for 30 minutes and later rinsed with distilled water and air dried overnight. For the preparation of gill slides, the molluscan specimens were pre-treated with 0.1% colchicine for 24 hours to arrest the metaphase stages. Then the snails were dissected and gill tissues were taken out and transferred to 0.07% hypotonic KCl solution and kept in it for 30 minutes. The tissues were fixed in freshly prepared carnoy's fixative (methanol and glacial acetic acid in the ratio of 3:1) and kept for 20 minutes giving three changes in it. Air drying dabbing method was used for preparing slides. Tissue was dabbed on clear and dry slides and eventually air dried. The slides were then stained in working solution of 4% Giemsa stain for 30 minutes and later rinsed with distilled water and air dried.

Scoring of MN slides

Micronuclei (MN) were identified according to the following criteria: (1) round and ovoid-shaped non-refractory particles in the cytoplasm, (2) colour and structure similar to those of chromatin, (3) a diameter of 1/3–1/20 of the main nucleus, (4) particles completely separated from the main nucleus (Fig. 2, 4) (Fenech *et al*., 2003). The frequency of micronuclei in atleast 1000 interphase cells/specimen (total of 4000 interphase cells from four specimens used per concentration and duration) and was expressed as mean±SD per 1000 cells. The micronucleated interphase cells from the two tissues were photomicrographed at 1000x magnification.

Statistics

Statistical analysis was performed by using the non-parametric Kruskal-Wallis test and the computer software 'PRIMERS-4.0'. $p<0.05$ was considered to be the level of significance. The frequency of micronuclei obtained from the experimental as well as controlled group was expressed as mean±SD.

RESULTS

Mercuric chloride treatment in gills (Fig. 2, 5)

Frequencies of micronuclei (MN) recorded in the gills after different treatments and exposure periods of mercuric chloride have been shown in table-1 and fig. 5. MN frequencies observed in mollusc treated *in vivo* with 4µg/l increased from 1.5±0.577 at 24h to 5.25±0.5 at 96h through 2.5±0.577 at 48h and 4.5±1.290 at 72h. Increased levels of MN were observed after all the exposure periods in experimental mollusc treated with 8µg/l. Maximum frequency of MN was obtained after exposure period of 96h (8.0±0.816) whereas minimum frequency was obtained after exposure period of 24h (2.25±0.957). In mollusc treated with 16µg/l of test chemical, frequency of MN was determined as 2.75±1.258, 4.75±0.5, 7.75±0.5 and 11.75±0.957 at 24, 48, 72 and 96h respectively. Similarly, the incidence of MN was found to increase in a time dependent manner from 2.0±0.816 (at 24h) to 13.25±0.957 (at 96h) after treatment with 32µg/l of copper. Data was found to be statistically significant versus respective controls after different exposure periods in all treatment doses (at 24h, $p\leq0.05$; at 48h, $p\leq0.01$; at 72h and 96h, $p\leq0.001$) as shown in table-1.

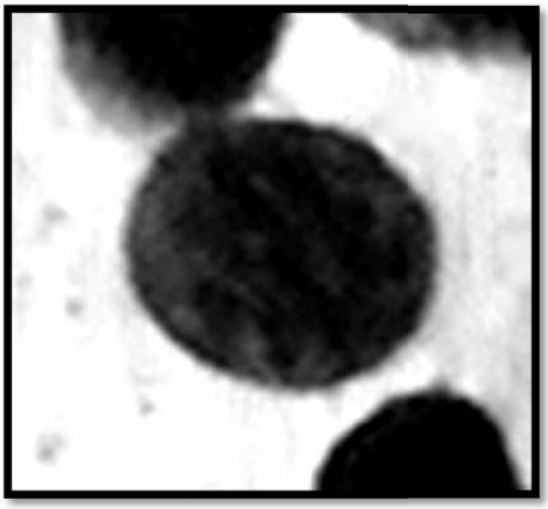

Fig. 1 Photomicrograph of gill cells of *B. bengalensis* with normal nucleus from control specimen.

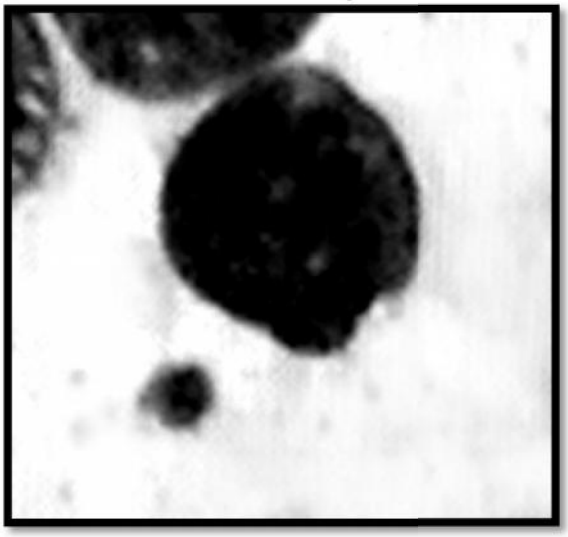

Fig. 2 Photomicrograph of gill cells of *B. bengalensis* with micronuclei from treated specimen.

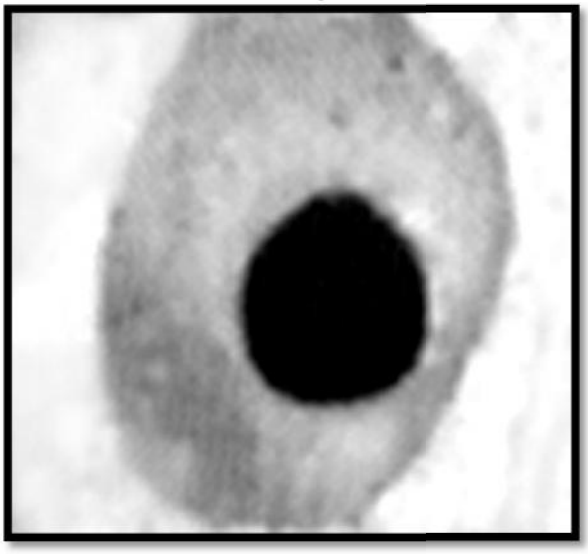

Fig. 3 Photomicrograph of haemocytes of *B. bengalensis* with normal nucleus from control specimen.

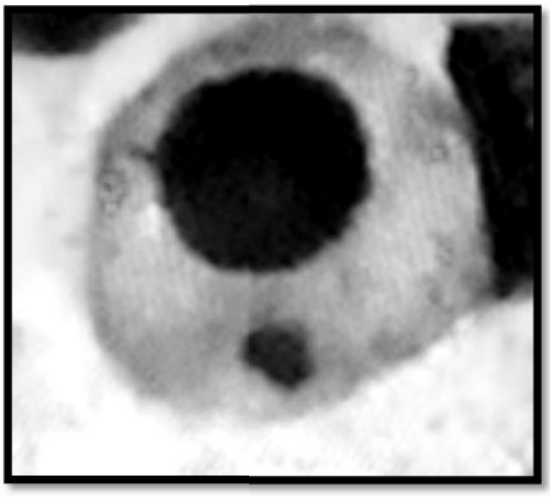

Fig. 4 Photomicrograph of haemocytes of *B. bengalensis* with micronuclei from treated specimen.

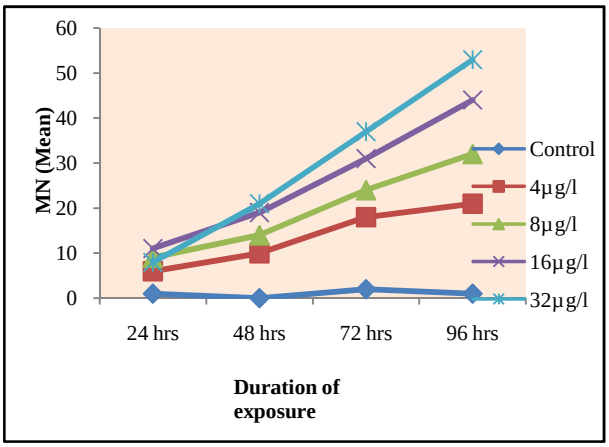

Fig. 5 Frequencies of micronuclei in gills after treatment with Mercury.

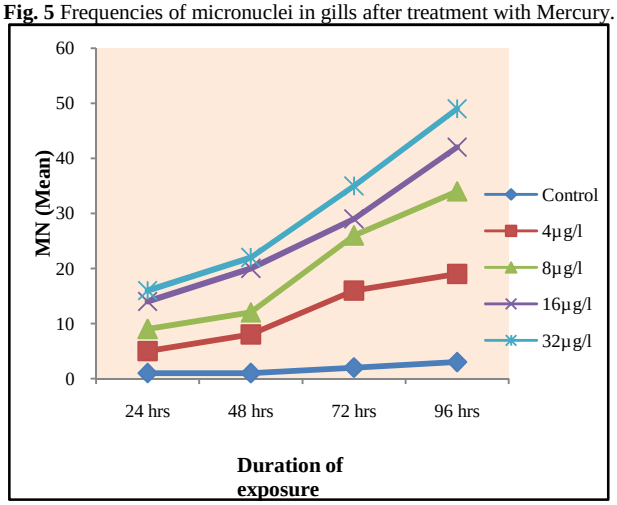

Fig. 6 Frequencies of micronuclei in haemolymph after treatment with Mercury.

Mercuric chloride treatment in haemolymph (Fig. 3, 6)

Table-1 and fig. 6 show the values of MN obtained in haemolymph after different treatments and exposure durations of mercuric chloride which were found to be statistically significant from the respective controls (at 24h and 48h, $p \leq 0.01$; at 72h and 96h, $p \leq 0.001$).

Table1 Frequencies of micronuclei (%) in gills and haemolymph of *Bellamya bengalensis* exposed to Mercury.

Conc. (µg/l)	Duration (hrs)	No. of specimens	Total interphase cells studied	Frequency of micronuclei (Mean±SD)	
				Gills	Haemolymph
Control	24	4	4000	0.25±0.5	0.25±0.5
	48	4	4000	0.0±0.0	0.25±0.5
	72	4	4000	0.5±0.577	0.5±0.577
	96	4	4000	0.25±0.5	0.75±0.957
4.0	24	4	4000	1.5±0.577[a]	1.25±0.5[b]
	48	4	4000	2.5±0.577[b]	2.0±0.0[b]
	72	4	4000	4.5±1.290[c]	4.0±0.816[c]
	96	4	4000	5.25±0.5[c]	4.75±0.5[c]
8.0	24	4	4000	2.25±0.957[a]	2.25±0.5[b]
	48	4	4000	3.5±0.577[b]	3.0±0.816[b]
	72	4	4000	6.0±0.0[c]	6.5±0.577[c]
	96	4	4000	8.0±0.816[c]	8.5±0.577[c]
16.0	24	4	4000	2.75±1.258[a]	3.5±1.0[b]
	48	4	4000	4.75±0.5[b]	5.0±0.816[b]
	72	4	4000	7.75±0.5[c]	7.25±0.5[c]
	96	4	4000	11.75±0.957[c]	10.5±1.290[c]
32.0	24	4	4000	2.0±0.816[a]	4.0±0.816[b]
	48	4	4000	5.25±0.957[b]	5.5±0.577[b]
	72	4	4000	9.25±0.5[c]	8.75±0.5[c]
	96	4	4000	13.25±0.957[c]	12.25±0.5[c]

[a] = p≤0.05, [b] = p≤0.01, [c] = p≤0.001, [ns] = non-significant (Kruskal-Wallis test; df=4)

Minimum (1.25±0.5) and maximum (4.75±0.5) values of MN were found to be induced after 24 and 96h of exposure respectively in treatment with 4µg/l of test chemical. In mollusc treated with 8µg/l of test chemical, frequency of MN was determined as 2.25±0.5, 3.0±0.816, 6.5±0.577 and 8.5±0.577 at 24, 48, 72 and 96h respectively. The incidence of MN was found to increase in a time dependent manner from 3.5±1.0 (at 24h) to 10.5±1.290 (at 96h) after treatment with 16µg/l of copper. At a sublethal concentration of 32µg/l, values of MN recorded at 24, 48, 72 and 96h were 4.0±0.816, 5.5±0.577, 8.75±0.5 and 12.25±0.5 respectively.

The data revealed that micronuclei frequencies increased with the increase in the doses and durations of mercury exposure in gills and haemolymph. Comparison between the micronuclei frequencies induced in tissues revealed the higher MN frequencies in gills than haemolymph.

DISCUSSION

Detection of micronucleus is a widely used parameter for cytogenetic damage. It is easily performed and also allows molecular approach in studying effects of clastogenic or aneugenic agents. As far as freshwater organisms are concerned, MN assay has been mainly applied to vertebrates (Jaylet *et al*., 1986; Chu *et al*., 1996; Hooftman and de Raat, 1982). However, freshwater snails are rarely used in such investigations. In the present study, gills and haemocytes of fresh water snail, *B. bengalensis* were used for detection of heavy metal genotoxicity by micronucleus test. Results of the present work showed that the treated snails have a significantly high frequency of micronuclei than that of the respective controls, suggesting the genotoxic effect of mercuric chloride. Comparison between the micronuclei frequencies induced in tissues revealed the higher MN frequencies in gills than haemolymph on account of gills being in direct contact with the test chemical in solution and therefore, were exposed to the highest *in situ* concentration of the compound.

The micronuclei frequency in control molluscs was negligible in relation to the exposed ones which could indicate the spontaneous origin of micronuclei in controls.

Cytogenetic damage has been described in molluscs inhabiting the marine port and oil terminal areas (Barsiene and Barsyte-Lovejoy, 2000; Barsiene, 2002). Significant differences in MN frequency have been described in gill cells and haemocytes of mussels exposed to different concentrations (50, 100, 500 and 1000 ppb) of benzo[a]pyrene for 48h (Venier *et al*., 1997). Scarpato *et al.* (1990) also studied the induction of micronuclei in the gill tissue of the mussel *Mytilus galloprovincialis* due to mutagenic activity of compounds vincristine (VCR) and benzo[a]pyrene (BaP) and found the micronuclei frequencies of sampled mussels to be significantly higher than the value of the control group. Hooftman and de Raat (1982) observed an increase in percentage of micronuclei with increasing concentration and duration of exposure to ethyl methane sulphonate in *Umbra pygmaea*. Similar observations were given by Pavlica *et al.* (2000) in haemocytes of zebra mussel, *Dreissena polymorpha* and great ramshorn snail, *Planorbarius corneus* exposed to pentachlorophenol (PCP). A dose dependent increase in micronuclei frequency was observed in haemocytes of both the species depicting haemolymph to be an appropriate test tissue in genotoxicity assessment. Haemolymph has been found to be the more appropriate test tissue in zebra mussel, *Dreissena polymorpha* than gills for environmental genotoxicity assessment (Mersch *et al.*, 1996). They accounted for several characteristics like a shorter preparation time of slides, a lower baseline MN frequency and a higher induction factor. On contrary, the present investigation reported gills to be more sensitive tissue than haemolymph as depicted by the micronuclei frequency (Table-1; Fig. 5, 6). There is a lot of variation in results regarding sensitivities of these cells in the micronuclei test. Fedato *et al.* (2010) also found gill cells to be more sensitive in bivalve mollusc, *Corbicula fluminea* than haemocytes which is in agreement with the present investigation.

In the present study, sublethal concentrations of mercuric chloride clearly increased the micronuclei frequencies which were dose and time dependent, compared to respective controls. The present results have been found to be similar to those reported by Al-Sabti (1994) who studied the micronuclei induction by mercury, selenium and methylmercury in the binucleated erythrocytes of Prussian carp. Cavas (2008) also observed the genotoxic effects of mercury chloride and lead acetate *in vivo* using the acridine orange stained peripheral blood erythrocytes, gill and fin epithelial cells of *Carassius auratus auratus*. MN frequencies in all three tissues were found to be elevated in fish exposed to both lead acetate and mercury chloride. MN elevation has been described in eel, *Anguilla anguilla* after treatment with cadmium and mercury (Sanchez-Galan *et al*., 2001; Teles *et al*., 2005). Rozgaj *et al.* (2005) have studied the genotoxicity of mercuric chloride ($HgCl_2$) in rats following oral exposure. They exposed female rats, aged 14 weeks, to $HgCl_2$ in oral doses of 0.068, 0.136 and 0.272 mg/kg body weight for five consecutive days and noticed that the micronuclei were significantly higher in the treated rats than in control. Present results are in agreement with the earlier studies carried out by Bolognesi *et al*. (1999) where the experimental studies on *Mytilus galloprovincialis* exposed to different concentrations of three heavy metal salts viz. $CuCl_2$, $CdCl_2$ and $HgCl_2$ were carried out and the genotoxic potential of copper, cadmium and mercury was obtained.

CONCLUSION

In conclusion, the results of the present study demonstrated high genotoxicity of mercuric chloride in fresh water mollusc species. Because of high sensibility and relatively low cost, use of MN as pollution biomarker in aquatic ecosystems can be useful. Studies needed to establish this biomarker in pollution effects on specific ecosystem. Exploration of the MN assay in fresh water molluscan species is welcome in order to improve the assessment of genotoxicity in aquatic and agricultural ecosystems.

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